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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68 — A1

(11) International Publication Number: WO 99/01581

(43) International Publication Date: 14 January 1999 (14.01.99)

(21) International Application Number: PCT/US98/13903

(22) International Filing Date: 2 July 1998 (02.07.98)

(30) Priority Data:
60/051,573 2 July 1997 (02.07.97) US

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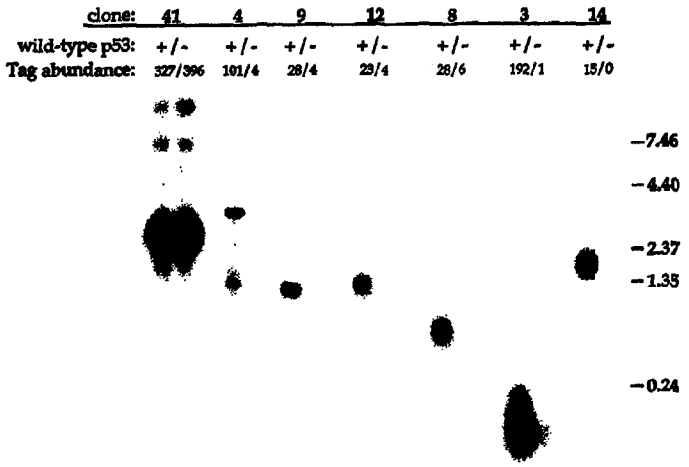
(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: P53 INFLUENCED GENE EXPRESSION



(57) Abstract

Serial analysis of gene expression (SAGE) allows for a quantitative, representative, and comprehensive profile of gene expression. We have utilized SAGE technology to contrast the differential gene expression profile in rat embryo fribroblast cells producing temperature–sensitive p53 tumor suppressor protein at permissive or non–permissive temperatures. Analysis of ~15,000 genes revealed that the expression of 14 genes ($p<0.001$, ≥0.03 % abundance) was dependent on functional p53 protein, whereas the expression of 3 genes was significantly higher in cells producing non–functional p53 protein. Those genes whose expression was increased by functional p53 include RAS, U6 snRNA, cyclin G, EGR–1, and several novel genes. The expression of actin, tubulin, and HSP70 genes was elevated at the non–permissive temperature for p53 function. Interestingly, the expression of several genes was dependent on a non–temperature–sensitive mutant p53 suggesting altered transcription profiles dependent on specific p53 mutant proteins.

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P53 INFLUENCED GENE EXPRESSION

TECHNICAL FIELD OF THE INVENTION

This invention is related to genes and proteins involved in cell cycle control and tumorigenesis.

BACKGROUND OF THE INVENTION

Transcriptional regulation mediated by the p53 tumor suppressor gene product is implicated in numerous cell regulatory cascades, most prominently cell growth regulation (White, 1996; Ko & Prives, 1996). While many genes have been shown to be transcriptionally regulated by p53 either directly (MDM2, $p21^{WAF1/CIP1}$, cyclin G, GADD45, IGFBP3, BAX, IGF-IR) or indirectly (Thrombospondin-1, TGF-α , PCNA, c-fos, c-jun, HSP70) (see Ko & Prives, 1996 for review), the cellular context required for specific p53-mediated transcriptional regulation remains ill-defined.

Rat embryo fibroblast (REF) cells transformed with activated RAS and a mouse temperature-sensitive p53 (Val135) gene constitute a tightly regulated, well defined system to study mechanisms involved in p53-mediated cell growth regulation (Michalovitz et al., 1990; Pietenpol et al., 1996; Martinez et al., 1991). Transformed REF cells grown at the non-permissive temperature (38°C) maintain the p53 protein in a non-functional conformation confined to the cytoplasm of the cell. Growth of RAS plus temperature-sensitive p53-transformed REF cells at the permissive temperature (32°C) results in the production of functional p53 protein

capable of migrating into the nucleus and regulating transcription in a sequence-specific manner (Velculescu et al., 1995; Gannon & Lane, 1991). Furthermore, a temperature shift from 38°C to 32 C induces functional p53-mediated G1 arrest and apoptosis (Velculescu et al., 1995). Both $p21^{WAF1/CIP1}$ (el-Deiry et al., 1993; Harper et al., 1993) and cyclin G (Okamoto & Beach, 1994; Zauberman et al., 1995) have been shown to be up-regulated in these cells by direct p53-dependent transcriptional regulation. Although the function of cyclin G remains undefined, $p21^{WAF1/CIP1}$ is known to regulate cell growth via direct interaction with cyclin-dependent kinases (CDKs) (Harper et al., 1993). Several other genes have been purported to be regulated by p53 within this system, but evidence for direct p53 transcriptional regulation is lacking (Ko & Prives, 1996).

Thus there is a continuing need in the art for discovering new genes which are regulated by p53 and genes which are related to cell cycle control and tumorigenesis.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods of diagnosing cancer in a sample suspected of being neoplastic.

It is another object of the present invention to provide an isolated and purified nucleic acid molecule which is identified by a SAGE tag.

It is an object of the present invention to provide an isolated nucleotide probe comprising at least 12 nucleotides of a rat nucleic acid molecule identified by a SAGE tag.

Another object of the invention is to provide a method for evaluating cytotoxicity or carcinogenicity of an agent.

These and other objects of the invention are achieved by one or more embodiments of the invention. In one embodiment, a method is provided for diagnosing cancer in a sample suspected of being neoplastic. The method comprises the steps of:

comparing the level of transcription of an RNA transcript in a first sample of a first tissue to the level of transcription of the transcript in a second sample of a second tissue, wherein the first tissue is suspected of being neoplastic

and the second tissue is a normal human tissue, wherein the first and second tissue are of the same tissue type, and wherein the transcript is selected from the group consisting of Alu, RAS, U6 snRNA,16S RNA, EGR-1, ribosomal protein S27, ETS-1, 28S RNA, CGR11, and LIMK-2;

categorizing the first sample as neoplastic when transcription is found to be lower in the first sample than in the second sample.

According to another embodiment of the invention a method is provided for diagnosing cancer in a sample suspected of being neoplastic. The method comprises the steps of:

comparing the level of transcription of an RNA transcript in a first sample of a first tissue to the level of transcription of the transcript in a second sample of a second tissue, wherein the first tissue is suspected of being neoplastic and the second tissue is a normal human tissue, wherein the first and second tissue are of the same tissue type, and wherein the transcript is identified by a tag selected from the group consisting of ribosomal protein L13a, α -tubulin (1), α -tubulin (2), thymosin β-4, and γ-actin;

categorizing the first sample as neoplastic when transcription is found to be higher in the first sample than in the second sample.

According to another aspect of the invention an isolated and purified nucleic acid molecule is provided. The nucleic acid molecule comprises a SAGE tag selected from the group consisting of SEQ ID NOS:11-16, 21-23, 25-28, 35-37, and 39-40.

In another embodiment of the invention an isolated nucleotide probe is provided. The probe comprises at least 12 nucleotides of a rat nucleic acid molecule, wherein the rat nucleic acid molecule comprises a SAGE tag selected from the group consisting of SEQ ID NOS:11-16, 21-23, 25-28, 35-37, and 39-40.

According to another aspect of the invention a method is provided for evaluating cytotoxicity or carcinogenicity of an agent. The method comprises the steps of:

contacting a test agent with a rat cell;

determining the level of transcription of a transcript in the rat cell after contacting with the agent; wherein an agent which decreases the level of a transcript identified by a SAGE tag as shown in SEQ ID NOS: 1-28, or an agent which increases the level of a transcript identified by a SAGE tag as shown in SEQ ID NOS:29-40 is a potential cytotoxin or carcinogen.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Cumulative total gene representation within the REF SAGE analysis. Sequenced SAGE tag (transcripts) accumulation was monitored for unique tags (genes) sporadically throughout the analysis using the SAGE software package.

Figure 2. Northern analysis of genes represented in the REF SAGE analysis. Poly A^+ RNA from 32°C, wild-type p53 (+) or 38°C, mutant p53 (-) REF-Val135 cells was electrophoresed, blotted, and probed with cDNA specific for clones 41 (EF1), 4 (cyclin G), 9, 12, 8 (ribosomal protein S27), 3 (U6 snRNA) and 14. Tag abundance obtained from the SAGE analysis is shown above the lanes. Molecular weight marker migration is depicted on the right (Kb, kilobases).

DETAILED DESCRIPTION

We describe here the use of serial analysis of gene expression (SAGE) (Velculescu et al., 1995) to provide an extensive profile of gene expression in REF cells containing non-functional or functional p53. We have identified novel p53 up-regulated genes and down-regulated genes previously undetected by EST, differential display, or subtractive hybridization technologies.

The genes which are identified as being upregulated or downregulated by p53 can be used to diagnose cancer in a sample. The sample can be a tissue sample isolated from a human which is suspected of being neoplastic. The level of transcription of an RNA can be determined and compared to the level in a normal

tissue, preferably of the same tissue source. Techniques for determining levels of transcription of an RNA are well known in the art, and include, without limitation, Northern blots, nuclear run-on assays, *in vitro* transcription assays, primer extension assays, quantitative reverse transcriptase-polymerase chain reactions (RT-PCR), and hybrid filter binding assays. These techniques are well known in the art. See J.C. Alwine, D.J. Kemp, G.R. Stark, *Proc. Natl. Acad. Sci. U.S.A.* 74, 5350 (1977); K. Zinn, D. Di-Maio, T. Maniatis, *Cell* 34, 865 (1983); G. Veres, R.A. Gibbbs, S.E. Scherer, C.T. Caskey, *Science* 237, 415 (1987).

When a transcript identified herein as being up- or down-regulated by p53 is found to be either up- or down-regulated in the test sample, then the sample can be categorized as neoplastic. Transcripts which are identified herein as being down-regulated in the absence of p53 are Alu, RAS, U6 snRNA, 16S RNA, EGR-1, ribosomal protein S27, ETS-1, 28S RNA, CGR11, and LIMK-2. Transcripts which are identified as being up-regulated in the absence of p53 are ribosomal protein L13a, α-tubulin (1), α-tubulin (2), thymosin β-4, and γ-actin.

To increase the reliability of the determinations, more than one of these transcipts can be assayed. Thus the level of at least two, five, six, or ten of the transcripts can be determined. Assays involving both up-regulated and down-regulated transcripts can be combined.

Also provided are new transcripts which have been identified herein on the basis of their up-or down-regulation in the absence of p53. These transcripts are identified by the SAGE tags shown in SEQ ID NOS: 11-16, 21-23, 25-28, 35-37, and 39-40. Given the SAGE tags is well within the skill of the art to isolate the RNA or cDNA which contains the SAGE tag. Due to the method of isolation of SAGE tags, they occur only in the 3' end of transcripts, immediately adjacent to the restriction enzyme site of the enzyme which was used to generate the SAGE tags. Thus hybridization under stringent conditions to cDNA libraries can be used to select larger cDNAs which contain the SAGE tag. While the SAGE tags isolated herein are derived from rat cells, similar sequences from other mammals, including humans, can be obtained using hybridization with the SAGE tags themselves, or using other portions of the rat genes identified by the SAGE tags.

Probes comprising at least 10, 12, 14, 16, 18, 20, 25, or 30 contiguous nucleotides of the cDNA identified by the SAGE tags can be used as probes. The probe may or may not contain the sequence of the SAGE tag. The probes can be labeled, as is known in the art, using for example, radiolabels, fluorescent labels, or enzymatic labels. These probes can be used to identify and isolate the homologues from related mammalian species. The probes can also be used to directly assay in humans or other mammals for up-or down-regulation in a sample of the corresponding transcript. Such regulation can be used as an indicator of neoplasia, as discussed above.

Combinations of probes can be provided in single or multiple vials as reagents for evaluating toxicity or carcinogencity of test compounds. The reagents can be provided in a kit, which optionally contains instructions for performing the assays, buffers, growth media, cells, detection reagents. In order to test compounds using such probes, the level of transcription of a transcript in a rat cell is determined after the rat cell has been contacted with the test compound. The effect of the test compound on transcription of the transcript identified by the probe is determined. Test compounds which cause changes in the transcript levels which mimic the changes caused by loss of p53 are identified as potential cototoxins or carcinogens.

We have applied SAGE technology toward the generation of growth regulatory transcript profiles from REF cells containing either functional or non-functional p53 protein. The profile includes over 15,000 genes derived from mutant and wild-type p53 cDNA populations. Statistically significant transcript differences between the two populations overwhelmingly favors the likelihood of a preferential transcriptional induction within functional p53 cell populations. This suggests that active transcription-dependent expression changes are responsible for growth arrest and/or apoptosis in this system. The number of apparently induced genes in the REF cells containing functional p53 might be an under-estimate since these cells have an intrinsically lower metabolic rate when grown at the lower permissive temperature, potentially slowing the accumulation of induced transcripts. Genes (actin and tubulin) required for growth were the ones with the most

disproportionately higher expression at the non-permissive temperature for p53. HSP70 also showed significantly higher abundance in cells lacking functional p53 protein, consistent with reports by others that it is down-regulated by wild-type p53 (Agoff et al., 1993).

Genes anticipated to be induced in the presence of functional p53 protein (cyclin G, $p21^{WAF1/CIP1}$, MDM2, BAX-1; Ko & Prives, 1996) did show different numbers of SAGE tags in the two libraries, however, a few anomalies where observed. First, the $p21^{WAF1/CIP1}$ transcript showed ~5-6 fold lower abundance than expected due to the proximity of a site for one of the restriction enzymes used in the generation of the SAGE libraries (see examples, below). Second, as noted in Table 2, one of the tags overexpressed in the 32°C REF cDNA library represents an internal cyclin G sequence. Since SAGE analysis relies on the 3'-most 4-base restriction endonuclease site for gene identification, the presence of two cyclin G tags (one 3' and one internal) suggests that either internal oligo(dT)-priming is occurring within the cyclin G transcript or there exists a second, previously unidentified, cyclin G transcript. Because sequences associated with the numerous other restriction sites in the cyclin G gene were not observed, it is unlikely that partial digestion of the cDNA can explain the "internal" tag. As can be seen in Figure 2, clone 4 (cyclin G) hybridizes to an RNA of the expected size and to a smaller (~1.2 kb) transcript. It is likely that this transcript represents an alternative form of cyclin G RNA that gives rise to the apparently "internal" SAGE tag. The combined abundance of the cyclin G transcripts is ~0-.34% of the total cDNA. While the significance of the two highly expressed tags and function of cyclin G remain undefined, such high levels of expression suggests cyclin G plays a major role in regulating cell growth of wild-type p53-containing REF cells. Finally, the presence of rRNA sequence tags results from the likely incomplete separation of mRNA from the rRNA population during library generation. Subsequent oligo(dT)-priming of dA-rich regions within specific rRNA's would result in rRNA-specific tags within the SAGE library.

Several potentially novel growth regulatory genes have been identified with this SAGE analysis including genes expressed to levels as high as 0.09% (clone 9)

of the induced mRNA population. All previously identified genes with the exception of U6 snRNA and LIMK-2 were previously found to be up-regulated in a p53-dependent manner including cyclin G (Okamoto & Beach, 1994; Zauberman et al., 1995), CGR11 (Madden et al., 1996), and EGR-1 and RAS (B. Vogelstein, personal communication). Whereas the RAS tag that was identified matches perfectly with the exogenous human RAS homologue, we cannot definitively exclude the rat RAS homologue as being responsible for the elevated tag levels since complete sequence information is not available for the latter.

The differential expression observed for U6 snRNA raises some interesting questions. Theoretically, the detection of this small RNA molecule should not be possible with oligo(dT)-dependent priming. While 3' modifications have been shown to occur to the U6 snRNA molecule, these modifications do not include base additions that facilitate oligo-dT priming (Lund & Dahlberg, 1992). One possible explanation for the observed differential detection of U6 snRNA is that upon apoptotic nuclear breakdown the U6 snRNA is liberated from the nucleus and fortuitously polyadenylated. No other snRNA species were detected in the SAGE analysis.

The EGR-1 transcription factor accounts for 0.1% of REF mRNA at 32°C. This well characterized transcriptional activator and repressor has been shown to be regulated in response to a wide array of growth regulatory stimuli, initially being described as an early growth response gene activated by serum (Sukhatme et al., 1988; Cao et al., 1990). Initial studies on EGR-1 appeared to correlate expression with enhanced cellular proliferation, however, more recently a role of EGR1 in cellular differentiation has been proposed (Bains, 1996), similar to the role proposed for $p21^{WAF1/CIP1}$ in myogenic differentiation (Liu et al., 1996). It is tempting to speculate that the EGR-1 induction observed in wild-type p53 REF cells stems from the triggering of molecular mechanisms similar to differentiation. Further, as some of the wild-type p53-containing REF cells are undergoing apoptosis, the identification of elevated levels of EGR-1 in these cells may indicate a more prominent role for EGR-1 in programmed cell death than previously appreciated.

SAGE analysis of REF-Phe132 control cells identified a small number of genes whose expression was apparently elevated in the REF-Phe132 mRNA population with respect to both 32°C and 38 C-maintained REF-Val135 cells (galectin-1 [Perillo et al., 1995], MTS1 [Ambartsumian et al., 1995], TRPM-2 [apolipoprotein J/clusterin] (Wright et al., 1996), osteopontin [Oates et al., 1996]). We have not confirmed these differences by other analyses. REF-Phe132-specific regulated genes could potentially represent unique transcriptional regulation dependent on specific p53 mutant proteins. Experiments by other investigators (Chen et al., 1994; Friedlander et al., 1996; Ludwig et al., 1996) have yielded results suggesting that specific p53 mutants interact with unique proteins hinting that p53 mutant proteins might retain transcription potential, but for genes not normally regulated by p53. It remains possible, however, that the observed differences result from leakiness of the p53 temperature-sensitive protein. That is, a subpopulation of "active" temperature-sensitive p53 protein at the non-permissive temperature might result in the selective down-regulation of genes subsequently observed to be preferentially expressed in the control mutant p53 population. It is noteworthy that we found significantly more divergence in the abundance of many SAGE tags when comparing REF-Phe132 versus 38°C REF-Val135 than when comparing REF-Val135 32°C versus 38°C. The former comparisons have been used previously for the identification of potential p53-regulated genes by differential display (Amson et al., 1996). Regardless, it is interesting that each of the known genes showing biased expression within REF-Phe132 cells has been linked to cellular growth regulation (Ambartsumian et al., 1995; Arai et al., 1996; Chambers, 1995; Guo et al., 1995; Perillo et al., 1995; Wright et al., 1996; Oates et al., 1996). Indeed, osteopontin was shown recently to be a metastasis-related factor in mammary tumors (Oates et al., 1996).

Although the results presented have provided a quantitative overview of potential growth-regulatory transcripts dependent on p53, the relatively limited public database of rat sequences compared to the human database precludes immediate identification of some of the differential tags identified. We have previously performed a differential display analysis of this REF p53 regulatory

system (Madden et al., 1996). Growth regulatory genes CGR11 and CGR19 were isolated, however the genes were found by random sampling. The isolation of these genes by differential display yielded little information regarding transcript abundance or relative importance to other p53-regulated genes. Identification of CGR11 and CGR19 in the current SAGE analysis demonstrates that while both were highly induced and moderately abundant, many other unknown genes shared similar characteristics that were not identified by differential display. Equally important, numerous genes appeared to be differentially expressed by differential display, but these apparent differences could not be substantiated by other criteria (*e.g.*, northern analysis).

Correlations of tag number with gene number suggest that many more than the identified 15,000 genes are expressed in REF cells. Indeed, recent SAGE analysis of ~60,000 transcripts in the yeast *Saccharomyces cerevisiae* resulted in the identification of nearly all of the anticipated 6,000 yeast genes (Velculescu et al., 1997). Estimates for expressed genes range from 10,000 to 50,000 unique mRNA's in a given cell type (Bains, 1996). Our REF SAGE analysis provides statistical confidence for all differential genes expressed at $\geq$0.03%. We expect that many other significant, differentially expressed genes will be revealed upon generation of further SAGE tags. The SAGE results presented here already provide a unique comparison in the transcript expression profile between cells harboring functional vs. non-functional p53 protein. Such broadly inclusive gene expression profiles are ultimately necessary and desirable for a thorough understanding of fundamental cellular processes such as growth regulation.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

Differential transcript profiles from temperature-sensitive p53 REF cells. Primary REF cells transformed with Ha-RAS and temperature-sensitive p53 (mouse Val-135) (REF-Val135) display growth arrest and apoptotic phenotypes as early as

4 hours after shift from the non-permissive to the permissive temperature (data not shown, see Ginsberg et al., 1991; Michalovitz et al., 1990; Madden et al., 1996). Control REF cells transformed with Ha-RAS and non-temperature-sensitive mutant p53 REF cells (REF-Phe132) do not exhibit growth arrest or apoptosis at the permissive temperature (32°C) (Ginsberg et al., 1991; Michalovitz et al., 1990). To provide a differential transcript profile inclusive of early p53-dependent transcriptional regulation, we harvested REF-Val135 cell mRNA 8 hours after shifting cells to the permissive temperature. Both cyclin G and $p21^{WAF1/CIP1}$ display strong transcriptional induction at this time, whereas little or no transcript is present in RNA harvested from REF-Val135 cells maintained at 38°C (non-permissive temperature) or from control REF-Phe132 cells shifted to 32°C for 8 hours (data not shown, see Madden et al., 1996).

EXAMPLE 2

Generation of SAGE and cDNA Libraries. Rat embryo fibroblast cells REF-Val135 (temperature-sensitive) and REF-Phe132 (provided by B. Vogelstein and M. Oren, see Ginsberg et al., 1991; Michalovitz et al., 1990) were maintained in DMEM containing 10% fetal bovine serum in 5% CO_2 at either 32°C or 38°C. Cells were trypsinized and replated at least 48 hours prior to any temperature shift. Temperature shifts were made by transfer of subconfluent flasks to pre-equilibrated incubators without media changes. RNA was harvested 8 hours after shift to 32°C. Total RNA was isolated by direct lysis in RNAzol (Tel-Test, Inc.). $PolyA^+$ RNA was isolated using the MessageMaker kit (Gibco/BRL) according to the manufacturer's instructions. SAGE libraries were generated using 2.5 ug $polyA^+$ RNA and the restriction enzyme NlaIII as described (Velculescu et al., 1995) except that the concatamers were cloned into SphI-digested pZErO-1 (InVitrogen). cDNA libraries were constructed using a λZapExpress system (Stratagene) according to the manufacturer's instructions. Hybridizations to λ clones were performed using either a 14 base or 15 base oligonucleotide end-labeled with ^{32}P (Velculescu et al., 1995). Some clones were obtained using the GeneTrapper kit (Gibco/BRL) and 15 base pair oligomers derived from the SAGE tag.

Plasmids. Rat clones for $p21^{WAF1/CIP}$ and MDM2 (provided by B. Vogelstein) were sequenced to determine respective SAGE tags. The 3'-most NlaIII sites are CATG/TATTTTGGTC and CATG/ATTTAGCAGT for $p21^{WAF1/CIP1}$ and MDM2, respectively. The rat $p21^{WAF1/CIP}$ sequence which juxtaposes the NlaIII and BsmFI sites is 5'-CATGTATTTTGGTCCC-3'. Adaptor ligation to NlaIII-digested rat $p21^{WAF1/CIP1}$ generates a second BsmFI site 5' to the endogenous BsmFI site ultimately resulting in a reduction of the $p21^{WAF1/CIP}$ tags observed in the SAGE library.

DNA Sequencing and Sequence Analysis. All DNA sequencing was performed with an ABI 377 (Applied Biosystems) automated DNA sequencer. SAGE clones were sequenced by first PCR amplifying pZErO-1 inserts with M13 forward and reverse primers followed by sequencing with Taq FS (Perkin Elmer) M13 -20 dye primer ready reaction mix. SAGE sequences were extracted and analyzed using the GenBank database (95.0) (containing 3450 and 224 rat mRNA and EST sequences, respectively) and the SAGE program software package. All other database analyses utilized the Wisconsin GCG package software programs (GenBank version 95.0). Statistical significance between samples was calculated using the equation:

$$(N_1 - kN_1^{1/2}) - (N_2 + kN_2^{1/2}),$$

where N_1 and N_2 represent the larger and smaller of the two numbers, respectively, and k is the degree of confidence; p=0.05 (k=1.96), p=0.01 (k=2.58), and p=0.001 (k=3.29). Positive values derived from the equation were deemed statistically significant at the respective confidence intervals.

SAGE tag abundance and differential expression. Data for more than 30,000 transcripts from each of 32°C and 38°C REF-Val135 cDNA were obtained by automated sequencing. An additional 10,519 transcripts analyzed from the control REF-Phe132 cells maintained at 32°C. A summary of transcript abundance and corresponding gene representation is provided in Table 1.

Table 1. Summary of sequence data from REF SAGE libraries.

SAGE library*	Tags†	GENES	
		Total	Multiple Occurances‡
REF-Val135 (32°C)	30,386	9,950	2,912
REF-Val135 (38°C)	30,313	9,240	3,086
REF-Val135 (32°C + 38°C)	60,629	15,562	5,301
REF-Phe132 (32°C)	10,519	5,119	1,231

* REF-Val135: REF cells transformed with RAS plus temperature sensitive p53.
REF-Phe132: REF cells transformed with RAS plus Phe132-p53.

† Cumulative numbers are presented for a combined analysis of sequences from the two (32°C + 38°C) Val135 libraries.

‡ ≥2 appearances of tag within library.

Sequences from each of the Val135 cDNA libraries represented more than 9,000 different genes, with about 3,000 being represented more than once in either library. Combined analysis of transcripts from both 32°C and 38°C REF-Val135 cDNA identified more than 15,000 different genes with more than 5,000 genes represented more than one time. Figure 1 details the increase in gene representation as the number of SAGE transcripts sequenced increases, demonstrating that many new transcripts are still being identified after 60,000 tags were sequenced. The transcripts identified from control REF-Phe132 cDNA (>10,000 SAGE tags) represent more than 5,000 genes with about 1,200 genes represented more than one time. Comparative analysis of 30,000 transcripts between 32°C and 38°C cDNA populations yielded 28 ($p<0.01$) and 14 ($p<0.001$) genes significantly up-regulated in cells expressing functional p53 protein (32°C) for transcripts present at an abundance level $\geq$ 0.03%. In contrast, the mutant temperature-sensitive p53 cDNA (38°C) population yielded only 12 ($p<0.01$) and 3 ($p<0.001$) genes differentially induced by comparison with the 32°C cDNA population. Twenty-two and 13 additional differential transcripts are apparent if statistical significance is relaxed to $p<0.05$ for elevated expression levels at 32°C and 38°C, respectively (0.02%-0.03% abundance). A summary of tags present at elevated levels ($p<0.01$) at 32°C and 38°C is presented in Table 2.

Table 2. Differential tag abundance in REF SAGE libraries.

CLONE	32°C	38°C*	TAG	abundance†	gene‡
REF-Val135 functional p53 up-regulated:					
1	786	497	GTGGCTCACA	2.62%	Alu
2	286	75	GAGGTGCCGG	0.95%	RAS
3	192	1	GCCCCTGCGC	0.64%	U6 snRNA
4	54	2	CTTTGGGTAC	0.18%	3' cyclin G
5	47	2	GGTTAGTTGG	0.16%	internal cyclin G
6	39	10	AATCAACCCG	0.13%	16S rRNA
7	31	1	GGATATGTGG	0.10%	EGR-1
8	28	6	CTCAGACAGT	0.09%	ribos. prot. S27
9	28	4	GGCCTGGCTA	0.09%	EST105829¶
10	25	2	GTGCTTGTGC	0.08%	ETS-1
11	25	4	TGCGGCCTCC	0.08%	NM
12	23	4	GTCCAGAGAC	0.08%	NM¶
13	21	0	CCACACCCTG	0.07%	NM**
14	15	0	AGTGTCCTGG	0.05%	NM¶
15	14	0	GAGATCAGTT	0.05%	NM
16	13	1	GAAGCTAATA	0.04%	NM
18	13	0	GGTCAGTCGG	0.04%	28S rRNA
19	12	0	ACCTTGGAGG	0.04%	NM
20	12	0	GGTATGGTGG	0.04%	CGR11
21	10	0	ATTGGCTGGG	0.03%	NM
22	10	0	GCCCTGCGCA	0.03%	NM
23	10	0	GGACTTTGTT	0.03%	NM
24	9	0	AGGCAGACTA	0.03%	LIMK-2
25	9	0	CAGGCTTCGT	0.03%	NM
26	9	0	CTGGGTTGGC	0.03%	NM
27	9	0	GCAGTCATCT	0.03%	NM
28	9	0	TTGACTCTTA	0.03%	NM
REF-Val135 functional p53 down-regulated:					
29	150	227	AGGTCGGGTG	0.76%	ribos. prot. L13a
30	48	103	ACGTCTCAAA	0.34%	α-tubulin(1)
31	42	83	TTGGTGAAGG	0.28%	thymosin β-4
32	25	79	GGTTGTTACT	0.26%	γ-actin
33	25	76	GCTGCCCTAG	0.25%	α-tubulin(2)
34	12	64	GAATAATAAA	0.21%	HSP70

35	12	39	TTCTGTGTGG	0.13%	NM
36	1	14	GTTCTGAACA	0.05%	NM
37	0	10	GAGACTTTGA	0.03%	NM
38	0	10	CAGGTGGGTG	0.03%	EST110550
39	0	9	CTGCCTTAAT	0.03%	NM
40	0	9	TGACAGTGAG	0.03%	NM

Miscellaneous tag abundance:

41	327	396	AGGCAGACAG	1.36%	EF1
42	107	92	GCCTCCAAGG	0.36%	GAPDH
43	46	32	CTACAGAGGA	0.15%	p53 (exogenous mouse)
44	18	8	GCAGACAGTG	0.06%	BAX(mouse)
45	15	13	GTGGCTGCTG	0.05%	cyclin D1
46	1	9	CAAACTGCAT	0.03%	CDK4
47	7	2	ATTTAGCAGT	0.02%	MDM2
48	5	0	TATTTTGGTC	0.02%	WAF1
49	5	0	GATGACGGGA	0.02%	CGR19
50	5	0	ATGACTCGTG	0.02%	NM**

* Underlines represent tags showing differentials with confidence $p < 0.001$.

† Percent abundance when induced.

‡ Mouse EST matches include: clone 12=MUSGS00660, clone 39=MUSGS00835

ǂ NM; no match

ϟ Represent genes for which cDNA and open reading frames have been obtained

**Clones identified by subtractive hybridization (B. Vogelstein and S. Zhou, personal communication)

The three 38°C elevated tags represent γ-actin (0.26%), α-tubulin (1 isoform, 0.25%), and HSP70 (0.21%) consistent with continuing growth of the 38°C-maintained cells. The remaining 38°C-elevated tags ($p < 0.01$) include a second tubulin isoform, Thymosin β-4, ribosomal protein L13a, and several undefined genes. Interestingly, CDK4 also showed a substantial differential induction in 38°C-induced cDNA (9 occurrences at 38°C and 1 occurrence at 32°C, $p < 0.05$).

The 14 genes expressed at elevated levels at 32°C ($p < 0.001$) include an alu repetitive tag (2.62%), RAS (0.95%), U6 snRNA (0.61%), 2 cyclin G tags (0.18% and 0.16%), EGR-1 [Zif268/NGFI-A/Krox-24] (0.10%), external transcribed spacer-1 (ETS-1) (0.08%), a clone previously identified by subtractive hybridization (clone 13; B. Vogelstein and S. Zhou, personal communication) (0.07%), 28S rRNA (0.04%), CGR11 (0.04%), and 3 uncharacterized genes (clones 9,12,14; Table 2). The genes corresponding to both cyclin G tags, U6 snRNA, and ETS-1 tags were cloned and verified to be expressed at the predicted SAGE abundance level (see below). The $p21^{WAF1/CIP1}$ tag was present at 5 copies in the 32°C cDNA and 0 copies in the 38°C cDNA. This apparent low abundance of the $p21^{WAF1/CIP1}$ tag (0.04% vs. 0.20% expected) is apparently due to the presence of a site for the restriction endonuclease BsmFI within the $p21^{WAF1/CIP1}$ cDNA that overlaps the SAGE tag site. Other genes showing elevated expression at 32°C include BAX1 (18:8), MDM2 (7:2), CGR19 (5:0), and another clone previously identified by subtractive hybridization (clone 50; B. Vogelstein and S. Zhou, personal communication) (5:0). One measure of reproducibility of the transcript profiles involves comparison of transcript levels for genes encoding ribosomal proteins and known housekeeping genes. As shown in Table 2 for EF1 (327:396) and GAPDH (107:92), well-known housekeeping genes are expressed at comparable levels in both samples. Most ribosomal proteins were also present at similar levels in 32°C and 38°C cDNA (data not shown). As expected, exogenous p53 (mouse Val135) also showed similar abundance in 32°C (46 tags) and 38°C (32 tags) cDNA populations (Table 2).

Partial cDNAs were obtained for three previously uncharacterized and relatively abundant rat tags at 32°C. The clone 14 partial cDNA yielded an open reading frame (ORF) of 210 amino acids showing strong homology within a

helix-loop-helix region to the HES transcription factor family (Sasai et al., 1992). Clone 12 yielded an ORF of 149 amino acids likely to be the rat homologue of a previously identified human tissue specific protein (GenBank accession #X67698) of unknown function and clone 9 yielded an ORF of 164 amino acids with no homology to published proteins or protein motifs.

EXAMPLE 3

SAGE data for control, non-temperature-sensitive REF cells. SAGE analysis of the control REF-Phe132 cDNA generated from 32°C-maintained cells was performed to greater than 10,000 transcripts. With a few exceptions, the transcript profile from these cells resembled that generated from the REF-Val135 38°C cDNA population (data not shown). One unknown gene was absent in the control REF-Phe132 cDNA but was expressed at about 0.10% in both the 32°C and 38°C REF-Val135 cDNA populations. Genes encoding galectin-1 (Perillo et al., 1995), MTS1 (Ambartsumian et al., 1995), TRPM-2 (apolipoprotein J/clusterin) (Wright et al., 1996), osteopontin (Oates et al., 1996), and one unknown gene were expressed to significantly higher levels in the control REF-Phe132 cDNA than in either the 32°C or 38°C REF-Val135 cDNA population (data not shown).

EXAMPLE 4

Northern blot analysis. RNA analyses were performed using either total or polyA$^+$ RNA and Ambion's NorthernMax kit. ^{32}P-labeled cDNA probes were generated by random-priming and hybridized and washed according to the manufacturer's protocol. Assurance of equivalent RNA loading was achieved either by UV shadowing (total RNA) or EF1 probing (poly A$^+$ RNA).

Validation of SAGE transcript representation. Confirmation of transcript abundance determined from the SAGE libraries was achieved using EF1 and cyclin G probe hybridization to 1 cDNA libraries derived from the same mRNA used for SAGE library generation. Both EF1 and cyclin G showed similar abundance in the SAGE and 1 cDNA libraries for both the REF-Val135 32°C and 38°C cDNA populations (data not shown). Northern analyses with clone 9, 12 and 14 cDNA as

well as probes for U6 snRNA, cyclin G, ribosomal protein S27, and EF1 were performed with mRNA derived from 32°C-and 38°C-maintained cells (Figure 2). Results show differential induction of all the unknown clones, U6 snRNA, cyclin G and ribosomal protein S27 in the 32°C mRNA population. As expected, the EF1 probe revealed equal abundance in both populations. Thus, all SAGE transcript differentials also show similar differential expression by northern analysis, confirming representative sampling in the SAGE analysis.

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CLAIMS

1. A method of diagnosing cancer in a sample suspected of being neoplastic, comprising the steps of:

comparing the level of transcription of an RNA transcript in a first sample of a first tissue to the level of transcription of the transcript in a second sample of a second tissue, wherein the first tissue is suspected of being neoplastic and the second tissue is a normal human tissue, wherein the first and second tissue are of the same tissue type, and wherein the transcript is selected from the group consisting of Alu, RAS, U6 snRNA,16S RNA, EGR-1, ribosomal protein S27, ETS-1, 28S RNA, CGR11, and LIMK-2;

categorizing the first sample as neoplastic when transcription is found to be lower in the first sample than in the second sample.

2. A method of diagnosing cancer in a sample suspected of being neoplastic, comprising the steps of:

comparing the level of transcription of an RNA transcript in a first sample of a first tissue to the level of transcription of the transcript in a second sample of a second tissue, wherein the first tissue is suspected of being neoplastic and the second tissue is a normal human tissue, wherein the first and second tissue are of the same tissue type, and wherein the transcript is identified by a tag selected from the group consisting of ribosomal protein L13a, α -tubulin (1), α -tubulin (2), thymosin β-4, and γ-actin;

categorizing the first sample as neoplastic when transcription is found to be higher in the first sample than in the second sample.

3. The method of claim 1 wherein a comparison of at least two of the transcripts is performed.

4. The method of claim 2 wherein a comparison of at least two of the transcripts is performed.

5. The method of claim 1 wherein a comparison of at least five of the transcripts is performed.

6. The method of claim 2 wherein a comparison of at least five of the transcripts is performed.

7. The method of claim 1 wherein a comparison of at least ten of the transcripts is performed.

8. The method of claim 2 wherein a comparison of at least six of the transcripts is performed.

9. A method of diagnosing cancer in a sample suspected of being neoplastic, comprising the steps of:

comparing the level of transcription of an RNA transcript in a first sample of a first tissue to the level of transcription of the transcript in a second sample of a second tissue, wherein the first tissue is suspected of being neoplastic and the second tissue is a normal human tissue, wherein the first and second tissue are of the same tissue type, and wherein the transcript contains an Alu sequence;

categorizing the first sample as neoplastic when transcription is found to be lower in the first sample than in the second sample.

10. An isolated and purified nucleic acid molecule which comprises a SAGE tag selected from the group consisting of SEQ ID NOS:11-16, 21-23, 25-28, 35-37, and 39-40.

11. The nucleic acid molecule of claim 10 which is a cDNA molecule.

12. The nucleic acid molecule of claim 10 wherein the SAGE tag is located at the 3' end of the molecule.

13. An isolated nucleotide probe comprising at least 12 nucleotides of a rat nucleic acid molecule, wherein the rat nucleic acid molecule comprises a SAGE tag selected from the group consisting of SEQ ID NOS:11-16, 21-23, 25-28, 35-37, and 39-40.

14. The probe of claim 13 which comprises the selected SAGE tag.

15. A reagent for evaluating toxicity or carcinogenicity of an agent, comprising at least 2 probes according to claim 13.

16. The reagent of claim 15 which comprises at least 5 of said probes.

17. The reagent of claim 15 which comprises at least 10 of said probes.

18. The reagent of claim 15 which comprises at least 20 of said probes.

19. The reagent of claim 15 which comprises at least 30 of said probes.

20. A reagent for evaluating cytotoxicity or carcinogenicity, comprising at least 2 probes according to claim 14.

21. A method for evaluating cytotoxicity or carcinogenicity of an agent, comprising the steps of:

contacting a test agent with a rat cell;

determining the level of transcription of a transcript in the rat cell after contacting with the agent; wherein an agent which decreases the level of a transcript identified by a SAGE tag as shown in SEQ ID NOS: 1-28, or an agent which increases the level of a transcript identified by a SAGE tag as shown in SEQ ID NOS:29-40 is a potential cytotoxin or carcinogen.

FIG. 1

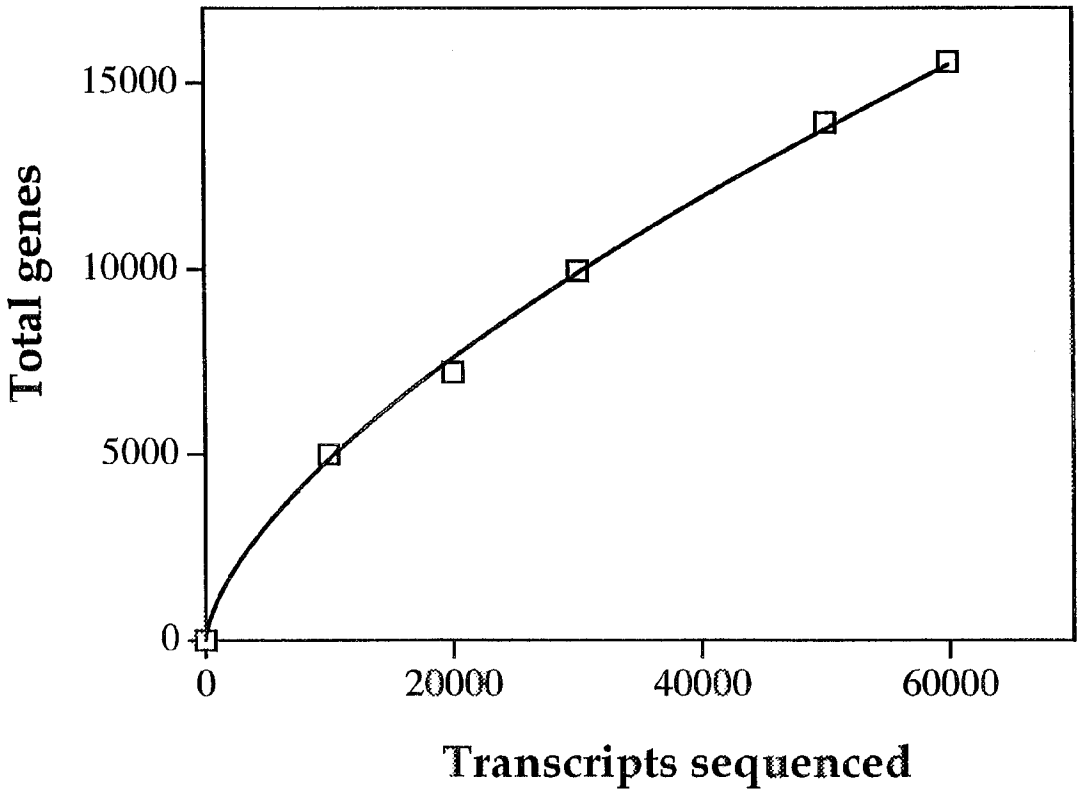
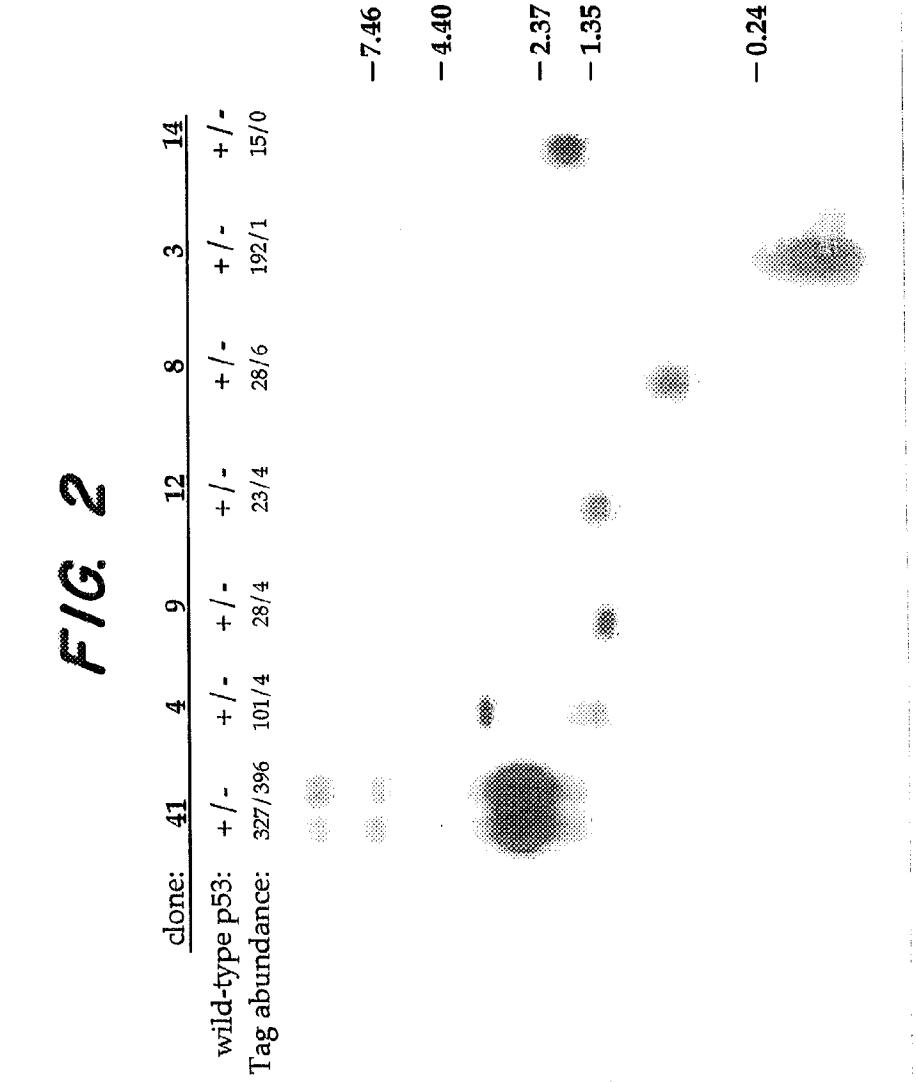

15000
10000
5000
0
Total genes
0
20000
40000
60000
Transcripts sequenced

FIG. 2


clone: 41 4 9 12 8 3 14
wild-type p53: +/- +/- +/- +/- +/- +/- +/-
Tag abundance: 327/396 101/4 28/4 23/4 28/6 192/1 15/0
−7.46
−4.40
−2.37
−1.35
−0.24

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/13903

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 97 45542 A (PHARMAGENICS INC) 4 December 1997 see claims 20-26 ---	1
P,X	MADDEN S ET AL: "SAGE transcription profiles for P53-dependent growth regulation" ONCOGENE, vol. 15, no. 9, August 1997, pages 1079-85, XP002083784 see table II ---	10-14
Y	ZHANG L ET AL: "Gene expression profiles in normal and cancer cells" SCIENCE, vol. 276, 23 May 1997, pages 1268-72, XP002083785 whole article, especially table 4 --- -/--	1-21

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
11 November 1998	27/11/1998

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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BEAUDRY G ET AL: "Therapeutic targetting of the P53 tumor suppressor gene" CURRENT OPINION IN BIOTECHNOLOGY, vol. 7, no. 7, December 1996, pages 592-600, XP002083786 see the whole document	1-21
Y	MADDEN S ET AL: "Induction of cell growth regulatory genes by P53" CANCER RESEARCH, vol. 56, no. 23, December 1996, pages 5384-90, XP002046025 see the whole document	1-21
Y	IOTSOVA V ET AL: "TATA-less promoters of some ETS-family genes are effeciently repressed by wild-type P53 " ONCOGENE, vol. 13, no. 11, December 1996, pages 2331-7, XP002083787 see the whole document	1-21
A	BUCKBINDER L ET AL: "GENE REGULATION BY TEMPERATURE-SENSITIVE P53 MUTANTS: IDENTIFICATION OF P53 RESPONSE GENES" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, October 1994, pages 10640-10644, XP002046023	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

I. ational Application No
PCT/US 98/13903

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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